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14. ABSTRACT
The purpose of the project is to investigate a potential application of ALT-711 (4,5-Dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride) in improving both mechanical and biological quality of femoral cortical bone for the purpose of providing better allograft materials via chemically breaking down AGEs (Advanced Glycation Endproducts) in the bone matrix. AGEs are naturally accumulated with age in connective tissues and have adverse effects on the biological and mechanical functions. Thus allografts sourced from bones with a high level of AGEs are likely to have a higher risk of nonunion and premature failure in the hosts. The current scope, as a proof of concept study, is to measure the effectiveness of ALT-711 on both cellular and mechanical characteristics of bones with and without prior glycation treatment (an artificial method to increase AGEs). We will measure effects of ALT-711 on osteogenic expression of stem cells on the bone substrates and also measure fracture toughness, a key measure of strong bone. We have demonstrated thus far ALT-711 has weak but measurable positive effects on the osteogenic expression of stem cells on bones without ribose treatment. We also have demonstrated ALT-711 is able to reverse the fluorescence level (assumed to be correlated to the amount of AGEs) previously increased by glycation. As such, we are encouraged by the preliminary data and now focusing our efforts in completing rest of the tasks assigned to the project. We expect to complete the work in the next period.

15. SUBJECT TERMS
Fracture healing, allograft bone, nonenzymatic glycation crosslinks, crosslink breaker, osteoconductivity, stem cell culture, fracture mechanics

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INTRODUCTION

Bone grafts are used to provide mechanical support and enhance the biological repair of skeletal defects. Age-related increase of advanced glycation endproducts (AGEs) within the collagen network of skeletal tissues adversely affects the mechanical and biological qualities of the allograft tissue. It is hypothesized that treatment with an AGE-breaker compound will increase fracture resistance and osteoconductivity of an allograft. Objectives are 1) to investigate the extent to which in vitro treatment of human cortical bone with an AGE-breaker will improve the fracture toughness of the tissue by treating normal and artificially glycated tissues in different concentrations of ALT-711 (4,5-Dimethyl-3-(2-oxo-2-phenylethyl)- thiazolium chloride) solutions followed by mechanical testing; 2) to investigate the extent to which in vitro treatment of young and old femoral cortical bone with an AGE-breaker will affect the recruitment, division and osteogenic development of mesenchymal stem cells by using normal and artificially glycated demineralized bone samples (treated or non-treated with ALT-711) in a cell culture. The degree of cell proliferation will be determined by ethynyl deoxyuridine incorporation, apoptosis by Tunel technique and osteogenic differentiation by Von Kossa staining for mineral deposition, alkaline phosphatase activity and expression of bone-characteristic genes, osteocalcin, Runx2, and col1a1 by RT-PCR. High-performance liquid chromatography and fluorescence microscopy will be used to quantify AGEs and crosslinks.

BODY

The following table summarizes the current state of progress in each task (**Table 1**).

Table 1. Summary of current work status

Tasks	Description	Progress	Status	Prerequisite
Task 1	Retrieval of 24 femurs	19 out of 24 femurs acquired	In progress	-
Task 2	Optimize AGE-breaker (ALT-711) treatments	Optimal ALT-711 concentrations and durations identified.	Completed ✓	-
Task 3	Preparation of test samples	17 femurs processed 15 femur-specimens ready for the mechanical testing	In progress	Task 1
Task 4	Mechanical testing	-	Not initiated yet	Tasks 1, 3
Task 5	Preparation of bone substrates for cell cultures	Preliminary test performed and analyzed. Protocol ready to be used.	In progress	Tasks 1, 3
Task 6	Measure cell division (ethynyl deoxyuridine)	-	Not initiated yet	Tasks 1, 3
Task 7	Measure apoptotic cell density (Tunel)	-	Not initiated yet	Tasks 1, 3
Task 8	Measure mineral nodules (Von Kossa/histochemical)	-	Not initiated yet	Tasks 1, 3
Task 9	Measure expression of osteogenic markers (alkaline phosphatase, osteocalcin, Runx2 and col2a1)	Preliminary tests performed and analyzed. Protocol ready to be used.	In progress	Tasks 1, 3
Task 10	Data analysis, publications, reports	Preliminary data analysis on effects of glycation (GLY) and ALT-711 (ALT) completed.	In progress	Tasks 1-9

Task 1. Retrieval of 24 cadaveric human fresh-frozen femurs from tissue banks and body donation programs. (Months 1-4.)

1a. Review and activation of tissue collection protocols by the donation program (Month 1).
1b. Collection and shipment of the femurs. (Months 1-4.)

Approval of the institutional review board (IRB 7511) for the project was obtained and submitted to the USAMRMC Office of Research Protections (ORP), Human Research Protection Office (HRPO) for the review along with a claim of exemption from the review to use preexisting cadaveric femurs. The statement that the project may proceed with no further requirement for review by the HRPO was received prior to the beginning of the supported period. In accordance with this notification (HRPO Log Number A-17384), newly retrieved femurs as well as previously existing deidentified cadaveric femurs are used.

As previously proposed, through approved tissue banks (the National Disease Research Interchange (NDRI) and Platinum Training) we have obtained 19 of 24 required femurs meeting age, sex and medical condition criteria. Those not yet procured are femurs of one young (65 years old or less) male, four young and one old female donors. The procurement of femurs has taken longer than initially planned as healthy donors are often prioritized for transplant surgeries. We have therefore revised some of the exclusion criteria taking account of a similar work done by Wu et al. [1] and feedbacks from the tissue bank (**Table 2**) so as to increase the chance of procurement without sacrificing the intention of the criteria.

Table 2. Old vs. new exclusion criteria. Exclusion criteria were revised to increase the chance of procurement and reduce miscommunication with the tissue providers without sacrificing the intention of the criteria.

	Original criteria	**New criteria**
Post mortem to inventory (PMI) time	24hr	72hr
Bed rest prior to death	Ventilated state	Over 6 weeks
History of radiation or chemotherapy	Any history of radiation or chemotherapy	Last five years before death
Medication	Anticonvulsant	-
Disease/conditions	-	Osteogenesis imperfecta Cushing's syndrome Syphilis

Task 2. Optimize AGE-breaker (ALT-711) treatments (36 sections/femur; 4 distal femurs. (Months 1-4.) - Completed

2a. Preparation of thin bone sections from femurs and demineralization in EDTA solutions. (Month 1.)
2b. Artificial glycation in ribose and ALT-711 treatment of the normal and glycated subgroups (0, 0.3mM and 3mM; 0, 5, 10, 20 hour groups). (Month 1-2.)
2c. Fluorescence analysis of AGE content. (Month 2-4.)

We have taken two approaches with the analysis of fluorescence, one using i) spectrophotometry and another using ii) fluorescence microscopy.

i) Spectrophotometry method

In order to relate fluorescence readings from the spectrophotometer to a known standard, a quinine reference standard was developed. Various concentrations of quinine were prepared in 100 ml sample volumes. A linear relationship between fluorescence and concentrations of quinine was found (**Figure 1**), which would allow presentation of arbitrary units of fluorescence in equivalent ng/mL quinine.

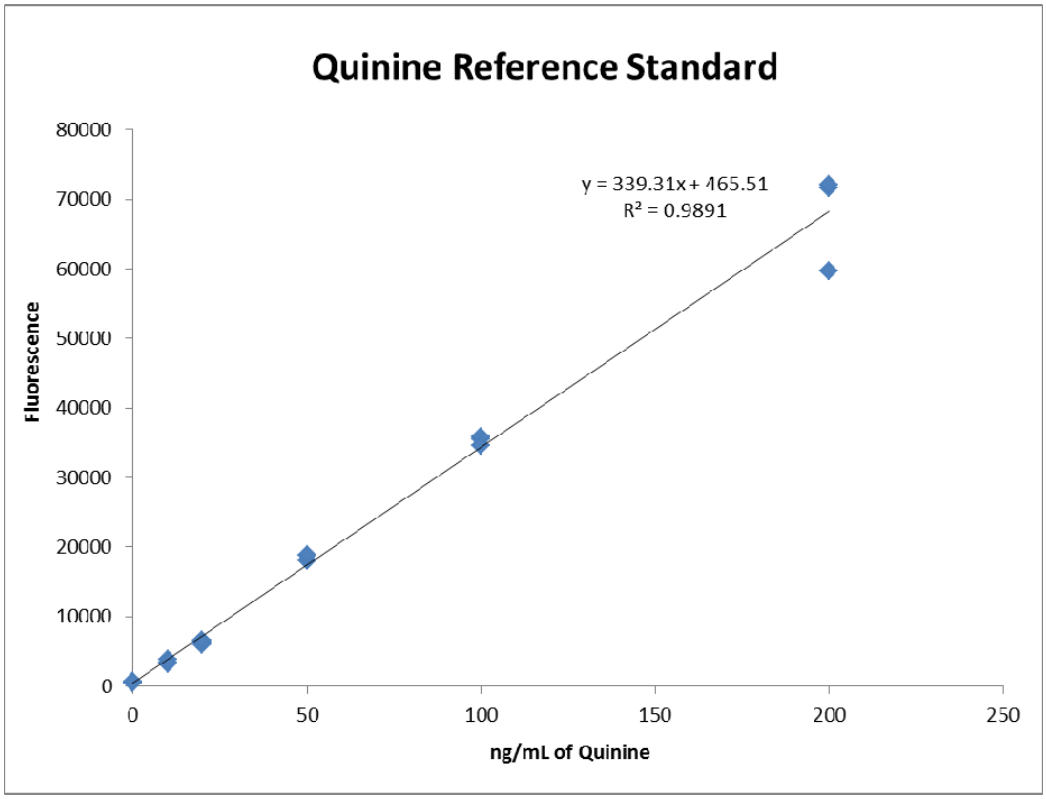

Figure 1. As expected, a strong linear relationship was established between arbitrary units of fluorescence and quinine concentrations. This relationship was intended to serve as a standard for quantification of fluorescence in our spectrophotometric approach.

For the initial studies to frame an appropriate range of bone powder concentration, ALT concentration, and treatment duration, approximately a ∅2x2cm section of a distal diaphysis was powdered to be used as a uniform reference. Our assumption was that the powder was sufficiently homogeneous to be used as a standard "bone" material. The decalcified specimens were digested in Papain (+ Tris Buffer) with 24 hours incubation at 55 ºC and prepared into 10, 25, 50 and 100mg powders in 1ml digestion solution. In order to determine the extent to which the digestion solution fluoresces, Papain + Tris buffer was also measured before and after 24 hour incubation at 55 ºC. We found that fluorescence and bone powder concentration had a linear relationship. All powder weights except 100mg were within the range of the quinine standard established above.

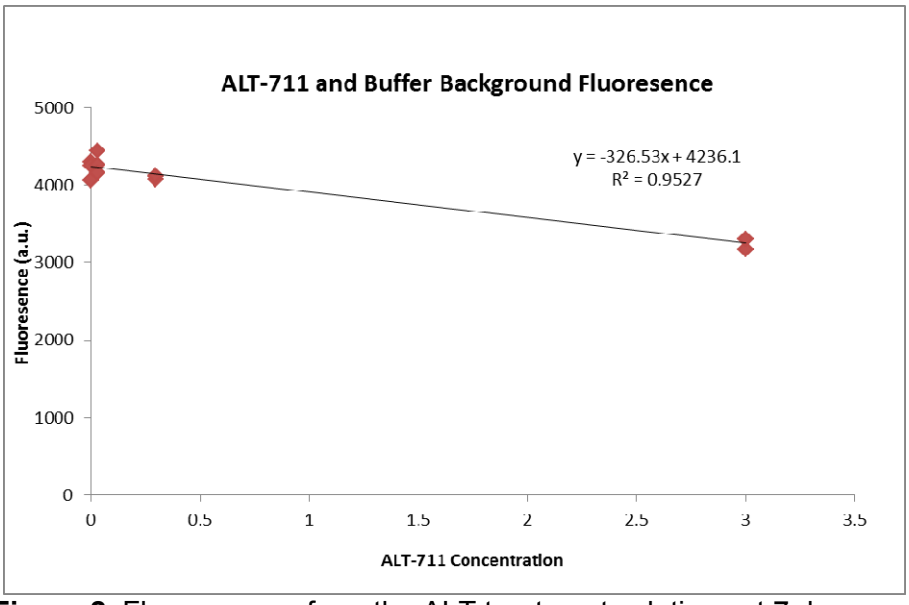

Figure 2. Fluorescence from the ALT treatment solutions at 7 days.

A slight decrease in fluorescence was observed with increasing ALT concentration (**Figure 2**). However, compared to bone, the background fluorescence occurred from the buffer and ALT treatment solution was small. We concluded that the fluorescence from the digestion or ALT solution is not high enough to prevent us from measuring variations in bone. The results from a series of experiments were consistent in that 3 mM ALT at 70 hours or more duration is an effective treatment (**Figure 3**), while less than 0.3 mM is not effective and 10 mM did not increase the fluorescence (**Figure 4**). In conclusion, 3mM for about one week appears adequate but the duration of the treatment must be controlled.

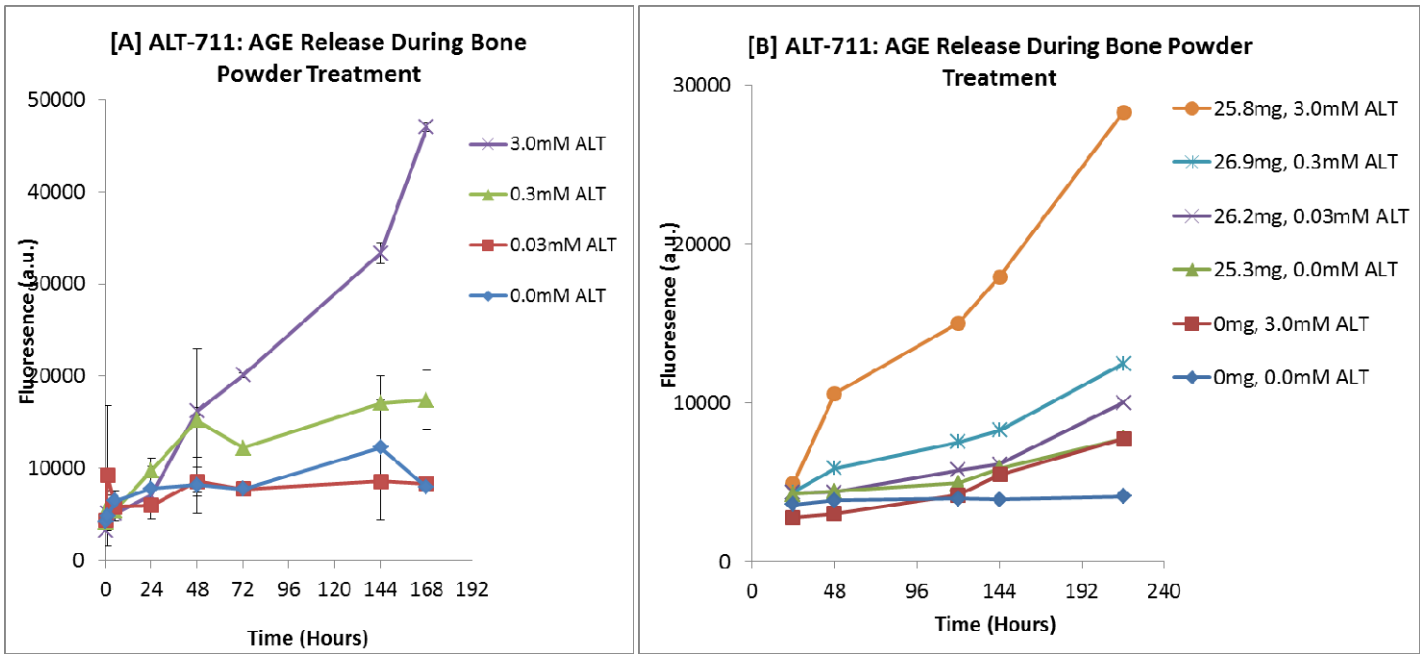

Figure 3 Results from typical series of experiments ((A) and (B)) in which the effect of time and dosage is examined. (A) These results suggest that at least 70 hours treatment in 3mM ALT is necessary to observe an effect. The error bars represent the standard deviation within triplicate measurements. (B) Another experiment using 30 mg bone powder, which is in general agreement with (A) that 3 mM is effective.

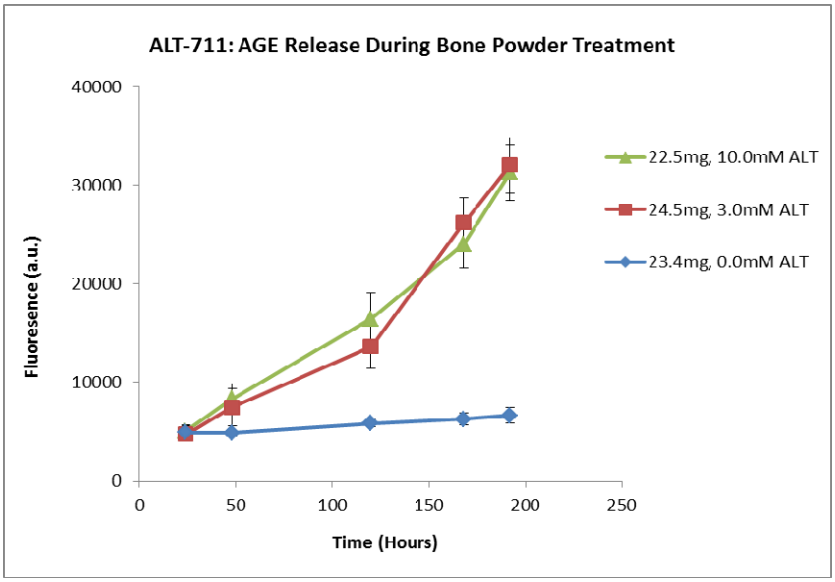

Figure 4. The experiment in **Figure 3B** was repeated with ALT concentrations of 3 mM and 10 mM to examine if the concentration of ALT was too low to be able to determine differences between the treatment concentrations. The fluorescence levels after 50 to ~200 hours were higher in treatment groups than no treatment but the 3 mM and 10 mM groups didn't appear different.

ii) Epi-fluorescence microscopy method

Epi-fluorescence microscopy method to detect relative changes in AGE concentration has two main advantages over the spectrophotometry method; i) it is a non-destructive method and thus a longitudinal observation of changes in AGE concentrations within each sample is possible, and ii) provides visual distribution of AGEs across the area of examination (**Figure 5**).

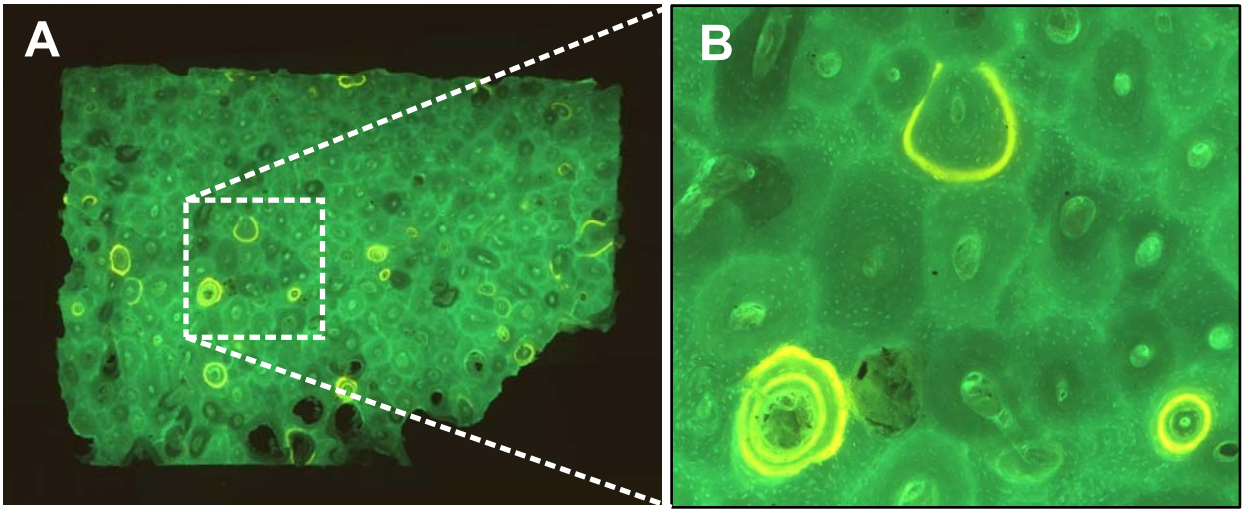

Figure 5. (A) ~100 µm thick human femoral bone section (undecalcified) under epi-fluorescence microscopy and (B) a subregion of the section showing a magnified view of the haversian system. Osteons with lower fluorescence are younger (recently formed) while those with brighter fluorescence are older (due to higher AGE content). Green-yellow concentric rings found around osteons are tetracycline residues in the bone, probably from antibacterial treatments while the donor was alive.

We prepared 20 femoral bone sections with thickness of 110 ± 15 µm using a circular diamond saw (South Bay, San Diego) under constant water irrigation to prevent heat damage to the tissue. A femoral bone piece (a 61 year old male donor) was aligned so that sections would reveal the axial plane (transverse to the supero-inferior direction) of the femur. The processed thin sections were further trimmed into a rectangular shape (approximately 5mm x 4mm) using a scalpel blade and a corner was also nipped to create a land mark for a reference for longitudinal imaging (**Figure 5**). The 20 sections were divided into five treatment groups (**Table 1**) and PBS buffer together with chloroform (20µL/25mL) and gentamicin (25 µL/25mL) to inhibit fungal and bacterial growth during 33 day of incubation (37 °C).

Before EDTA decalcification, they were individually wet mounted and photographed under epi-fluorescence microscopy (Nikon Corp., Tokyo, Japan) using a x4 objective with 60ms exposure time. The light source was ultraviolet (UV) through 400±440-nm excitation and 480-nm barrier filters. Then sections were decalcified for 50 hours and then another set of images were taken for day 0 reference. Logitudinal flurorescence imaging was performed every second to third day up to two weeks and then weekly thereafter up to 33rd day (**Figure 6**).

Table 3. Description of the treatment groups. Each treatment group included 4 sections.

Treatment group notation	Followup treatment	Description	Treatment solution	Storage temp	Duration
GLY	GLY → ALT(n=2) GLY → PBS(n=2)	Initially glycated only but later divided into two groups GLY→ALT and GLY→PBS at day 22.	Ribose (666mM) ALT-711 (3mM) PBS	37 °C	GLY = 22 days ALT = 11 days PBS = 11 days
GLY+ALT		Glycated for 7 days and then ALT	Ribose (666mM) ALT-711 (3mM)	37 °C	GLY = 7 days ALT = 26 days
ALT		ALT-711 only	ALT-711 (3mM)	20°C	33 days
C		Control – no treatment	PBS	20°C	33 days
F		Frozen control – no treatment and kept in a freezer	PBS	-20 °C	33 days

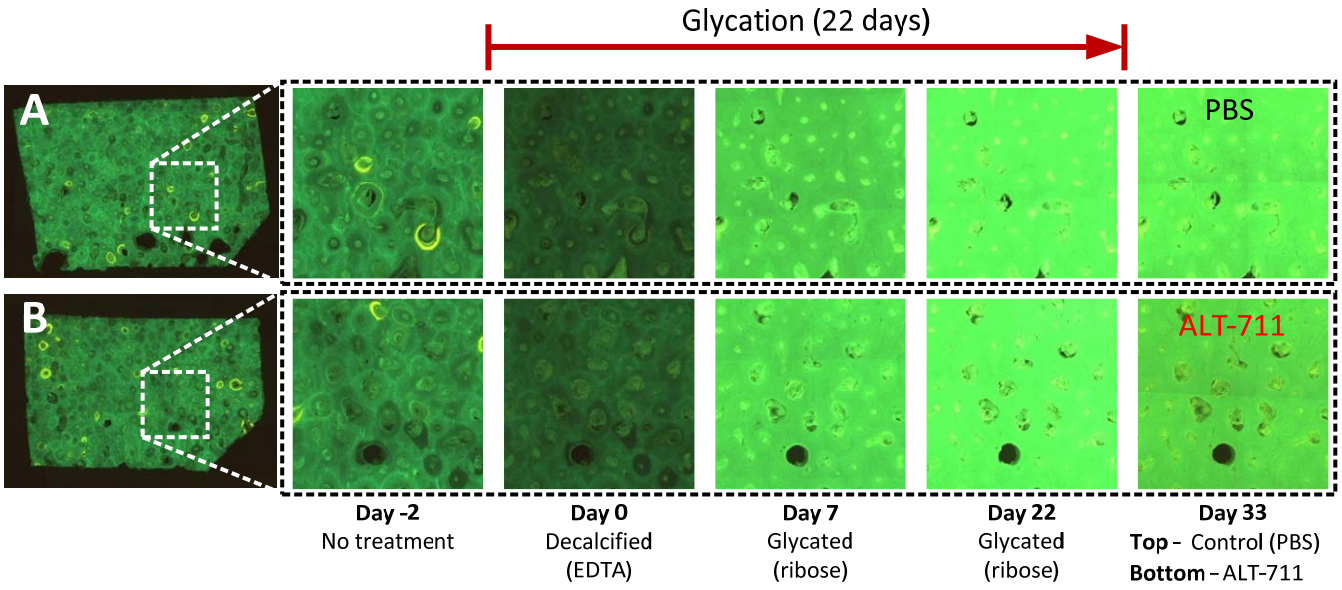

Figure 6. Longitudinal changes of fluorescence level (FL) from decalcification, glycation and ALT-711 treatments. Both (A) and (B) show steady increase of FL during glycation upto 22nd day. The FL is lower after 11 days of ALT-711 treatment than at 22nd day of glycation (B)(A) while no significant difference was observed after 11 days of PBS treatment (control) following the 22 day glycation regimen (A).

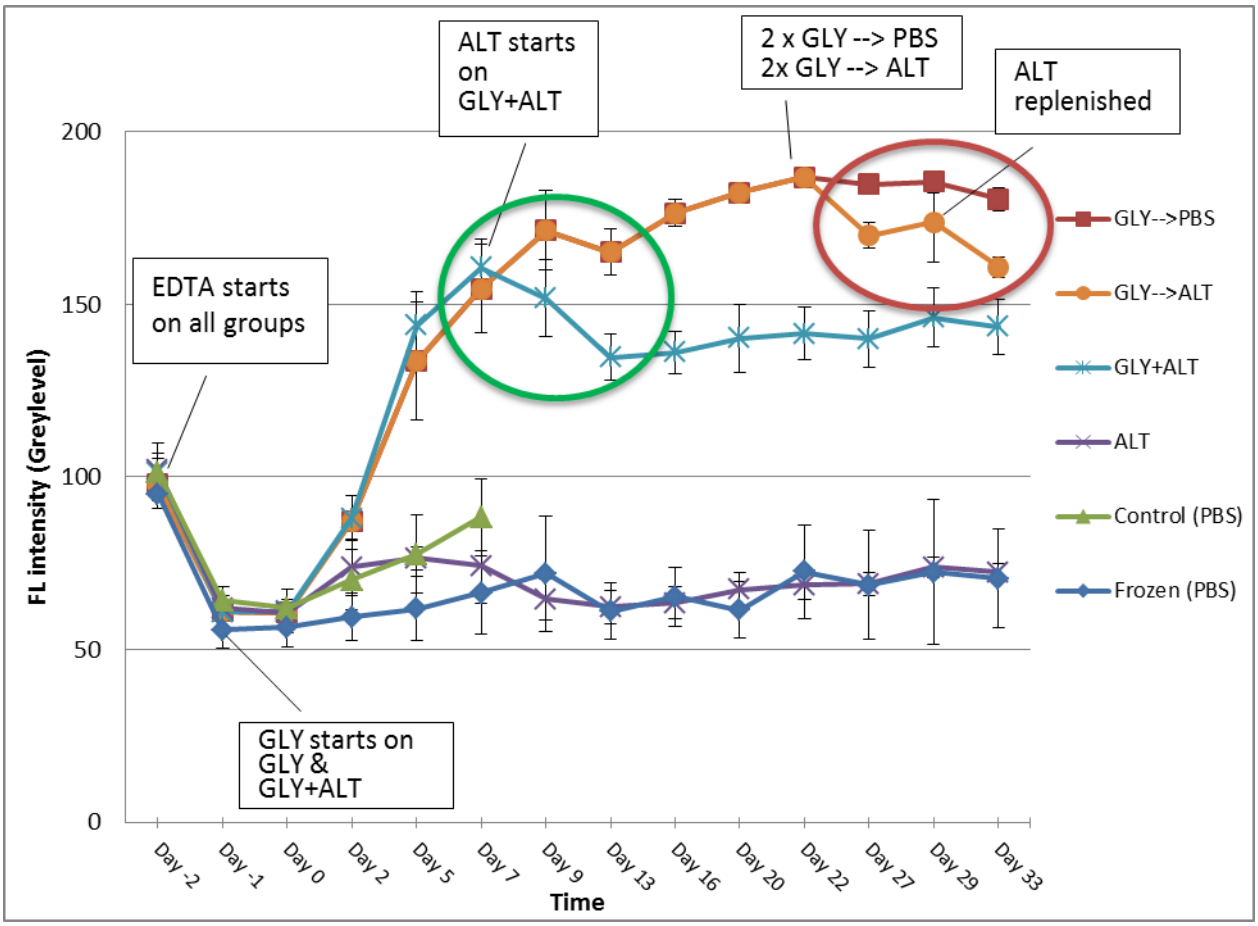

Figure 7. Longitudinal changes in averaged fluorescence level (FL) over the sample in each group from decalcification, glycation and ALT-711 treatments. A marked drop in FL can be observed for the GLY+ALT group during the period from day 7 to 13 (green circle) and for the GLY→ALT group during the period from day 22 to 33 (red circle). Error bars indicate standard deviations.

We observed a rapid increase in fluorescense levels in both GLY and GLY+ALT groups (**Figure 7**) upto day 9 and begining to plateaue at day 22. When ALT-711 was introduced in the GLY+ALT group at day 7, the increase in FL from the previous glycation treatment was reversed, reducing FL by 15%. However after day 13, FL a slow but steady increase could be observed again. In GLY specimens, ALT-711 treatment was introduced to 2 of 4 GLY sections at day 22 while the other 2 sections were placed in PBS solution to confirm the ALT's effect in reducing FL observed in the GLY+ALT group between day 7 and 13. At this point, all four GLY sections were at a nearly saturated state from 22 days of GLY treatment. Upon ALT-711 treament on the GLY saturatrated sections, the FL decreased at day 27 and then further decrased up on a replenishment of ALT-711 solution at day 29, resulting in a total decrease of 14%.

The ALT group, where ALT-711 was immediately applied to freshly decalcified sections, did not show a decrease in FL; rather, there was a small but steady increase in FL identical to the frozen control group (F). Control group incubated at 37 °C also had a steady increase in FL but not to to the extent of that in the glycated groups, indicating time is also a factor in FL increase in the sections.

In order to confirm the ability of ALT-711 to reverse the increased FL by ribose glycation, we conducted another similar experiment with 40 bone sections, allocating 10 sections to each group (i.e. GLY→PBS, GLY→ALT, ALT, PBS control). In this experiment all four groups were subjected to a 37 °C incubation condition as opposed to the previous experiment, in which the ALT and control groups were kept in room temperature. As in the previous experiment, we observed a marked decrease (-20%) in FL levels that were initially raised by glycation (GLY→ALT, $p < 0.0048$) relative to the group that was treated with PBS after glycation (GLY→ PBS; control). Additionally, no substantial differences were observed in the ALT and control groups over the 14 day period (**Figure 8**)

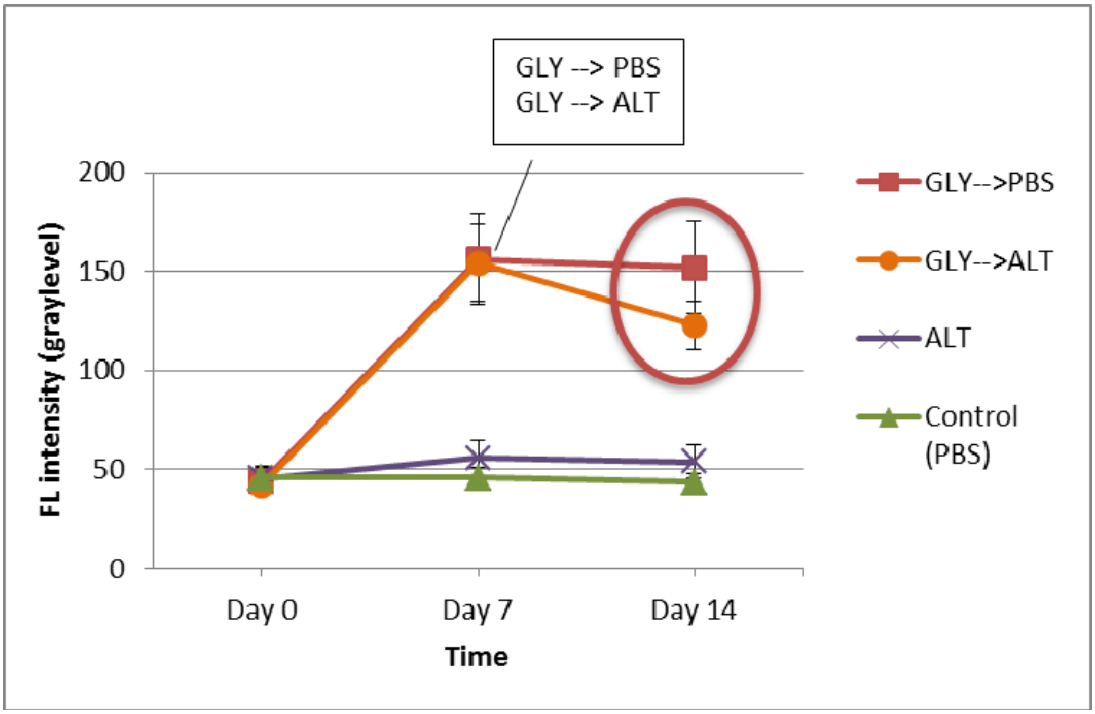

Figure 8. Both GLY→PBS and GLY→ALT groups have identically increased their FL at day 7 by glycation and there is subsequent decrease from ALT-711 in GLY→ALT while no significant change is seen in GLY→PBS. Both ALT and PBS control groups did not change their FL significantly over the 14 day period. This experiment confirms that ALT-711 has a significant reducing effect on FL in previously glycated samples.

The results of both experiments suggest that ALT-711 of 3 mMol concentration over 7 day application has a significant effect on reducing the level of fluorescense intensity (i.e. AGE content) previously increased by ribose glycation ($p < 0.0048$). However after day 7, its potency is reduced and further replenishment of the solution was needed. This suggests that 3mMol of ALT-711 has a potent duration of approximately 7 days but its effect is cumulative and can be continued by replenishing the solution. Based on the projection on the FL gradients in **Figure 7** and **Figure 8**, over 30 days of ALT-711 treatment may reverse signifncant amount of FL gained by the glycation process.

Stability of pH over incubation period

Due to potential acidification of treatment solution which can lead to unwanted decalcification of bone specimens during a long incubation period, we investigated whether ribose or ALT-711 solutions would maintain a neutral pH level (i.e. 7) over a 14 day incubation period at 37 °C. We prepared 25ml of seven treatment solutions (**Table 4**) with combinations of PBS, ribose (667 mMol), ALT-711 (3 mMol) and $CaCl_2$ (57.5 mg/liter), which is used to prevent calcium leaching from bone in a long incubation period [2].

Table 4. Description of the solutions and pH values over 2 week period at incubated in 37 °C. The three ribose containing treatment groups (GLY, GLY+CaCl2, GLY+ALT) are substantially acidified in 14 days (marked by *) decreasing their pH from 7 to 4.32.

			pH measured		
Treatment group	**n**	**Treatment solution**	**Day 0**	**Day 7**	**Day 14**
Control	1	PBS	7.01	6.99	7.02
$CaCl_2$ control	1	PBS+$CaCl_2$	7.01	6.96	6.99
ALT	1	ALT-711+PBS	7.01	6.85	6.72
ALT+ $CaCl_2$	1	ALT-711+PBS+$CaCl_2$	7.01	6.87	6.74
GLY	2	Ribose+PBS	6.86	5.28	4.32*
GLY+ $CaCl_2$	1	Ribose+PBS+$CaCl_2$	6.82	5.42	4.35*
GLY+ALT	1	Ribose+ALT-711+PBS+$CaCl_2$	6.91	5.63	4.47*

We found marked decrease in pH in all GLY solutions from the initial pH 6.91 to 4.32 by the end of Day 14 (Figure 9). However there was only minor decrease of pH level in ALT solutions. We also observed CaCl2 solutions formed white precipitate with PBS (likely to be cacium phosphate), however this caused no discernable change to the pH.

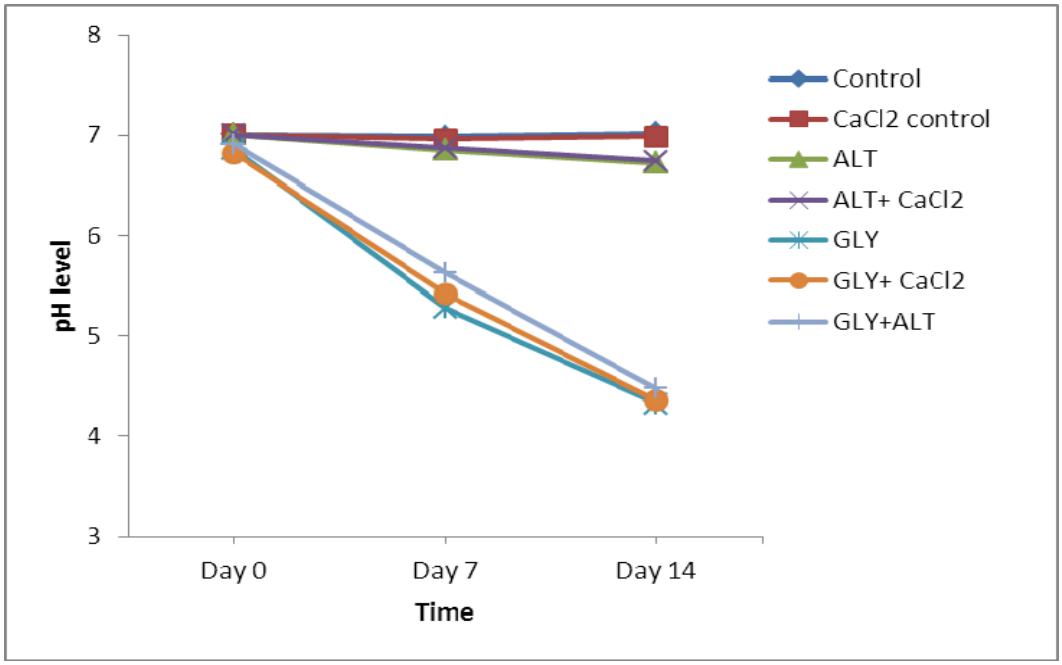

Figure 9. pH level change during a 14 day incubation period (37 °C) of ALT-711 (ALT) and ribose (GLY) solutions with and without $CaCl_2$. Any treatment solutions containing ribose (GLY) acidified quickly turning into a mild acid in two weeks.

In order to see whether daily replenishment of ribose would prevent severe acidfication, we again conducted an experiment but measured daily changes in pH in both control (PBS) and ribose solution. After one day, control-adjusted pH decrease in ribose solution was 0.51, which indicates daily replacement of freshly made ribose solution would restrict range of acidification and likely to minimize the undesired decalcification effect. Since the precipitation formation between $CaCl_2$ and PBS is not easily preventable and has no significant effect over pH level, we have decided not to use $CaCl_2$ in the buffer in the future.

Task 3. Preparation of test samples from 24 femurs. (Months 2-8.)

3a. Machining of 4 compact tension test specimens per femur using a CNC micromilling machine. (Months 2-6)
3b. Artificial glycation in ribose and AGE-breaker treatment of the normal and glycated subgroups (Months 7-8)

We have used a CNC machine (Denford Micromill 2000, West Yorkshire, UK) to machine compact tension (CT) specimen based on a previous work by Brown [3]. While Brown have used saline dripping irrigation method to prevent over heating of bone during machining, we have decided to machine the bones specimens submerged in a saline bath to prevent any chance of over heating that may inadvertently degrade collagen matrix (**Figure 10**).

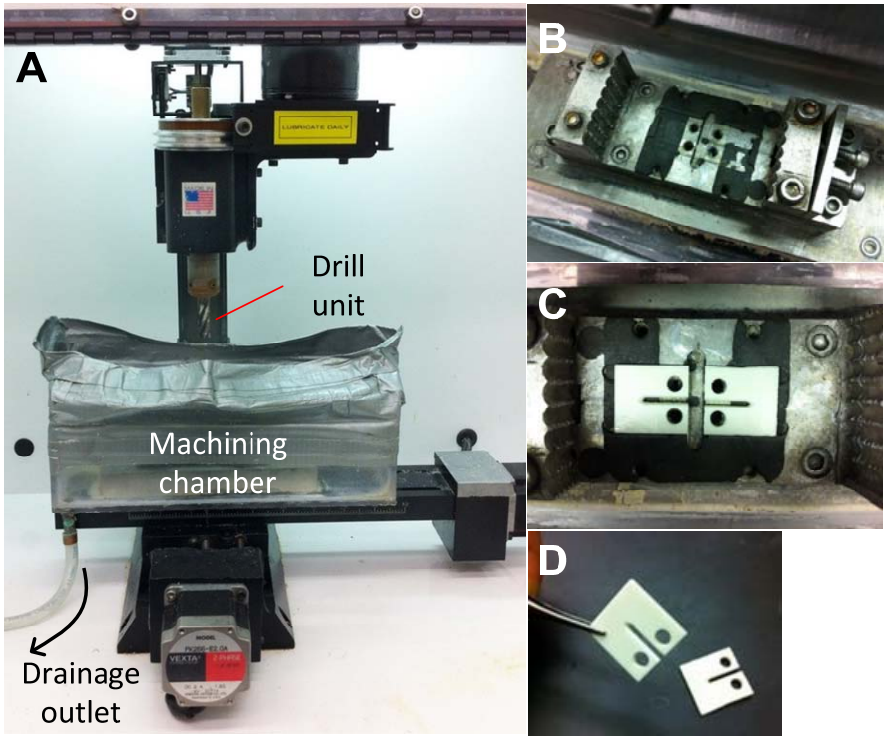

Figure 10. (A) CNC setup for compact tension (CT) specimen machining where the machining chamber was filled with saline during the entire machining process. The chamber contains (B) a specimen vice to grip the femoral section during the initial cutting process and (C) a die platform to hold the CT specimens during machining of the finer details.(D) Typical fully machined CT specimens.

Machining CT specimens fully submerged underwater required remanufacturing of the CNC machining station to incorporate a saline bath chamber, a mini vice and a drainage system. Furthermore, the CNC machining program was rewritten to account for special restrictions from machining underwater.

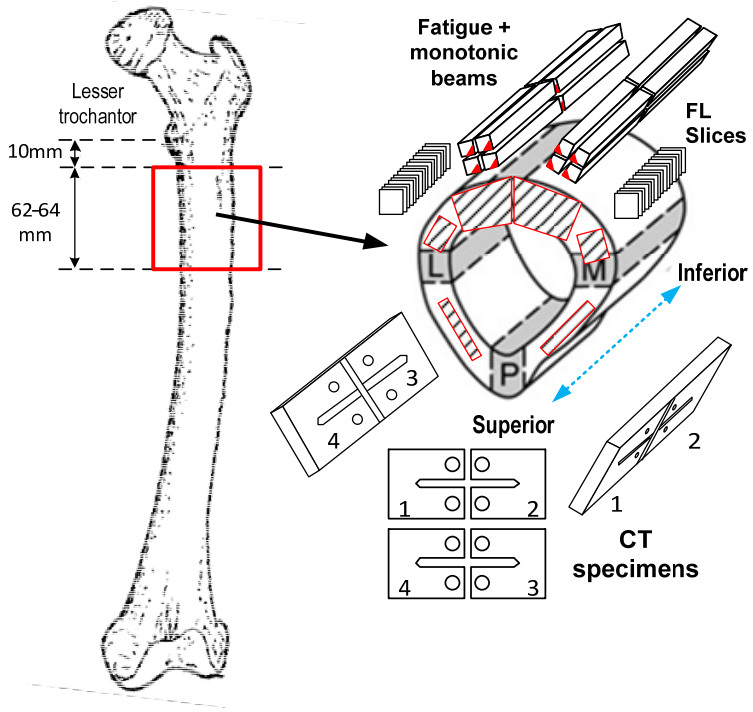

Figure 11. Cutting scheme of a femur. A 62mm to 64mm section was cut 10mm below the lesser trochanter from each femur. The section was divided between medial and lateral segments and then CNC machined to produce four CT specimens from the proximal-medial and proximal-lateral cortices. The remaining bone pieces were used to cut fatigue/monotonic test beams and 100 μm thick bone slices for the osteogeneity experiments.

From each femur, we have sought to obtain as much samples as possible (**Figure 11**). 4 CT specimens were obtained from medial-proximal and lateral-proximal facets while remaining bones were used to cut out 2x2mm beams and 100μm thick bone slices. We have processed 17 femurs in this manner; however, two femurs had cortices too thin or too porous to machine and thus were rejected in the end. The CT specimens will be used to test fracture toughness while the beams will be kept as contingent mechanical test samples. The bone slices will be used as bone substrates for the stem cell osteogenic experiments as planned.

Task 5 and Task 9. Preparation of bone substrates for cell cultures from 24 femurs. (Months 4-7.) + Measure expression of molecular markers of mineralization, osteocalcin, Runx2 and col1a1 using quantitative RT-PCR with specific primers. (Months 14-15.)

5a. Preperation of 100 μm-thick sections (triplicates per measurement) from the middiaphysis. (Months 4-5.)
5b. Demineralization of bone sections in EDTA solutions. (Months 5-6.)
5c. Artificial glycation in ribose and AGE-breaker treatment of the normal and glycated subgroups. (Months 6-7.)
5d. Culture human mesenchymal stem cells (Lonza, Walkersville, MD) on bone sections. (Months 7-14.)
Milestone 5: The effect of AGE-breakers on osteogenic differentiation of stem cells is determined.

The purpose of this preliminary investigation was two-fold;

1) to verify whether the current proposed culture protocol was able to induce osteogenic differentiation in the stem cells on treated bone substrates,

2) to examine whether there is a measurable improvement in osteogenic expression markers by ALT-711 treatment in bone sections that has naturally accumulated AGEs (i.e. control-PBS vs ALT) and in bone sections with artificially accumulated AGEs (i.e. GLY vs GLY+ALT).

We have measured four gene expression markers (**Table 5**) that indicate the activity and stage of cell differentiation and then compared with the known temporal gene epxression patterns from the literature. And then using those relative levels of gene expression markers, we statistically compared between four groups; i) ALT vs PBS and ii) GLY+ALT vs GLY at day 7 and 14.

Table 5. Summary of osteogenic markers used in this study.

Osteogenic marker	Description
Alkaline phosphatase (ALPH)	Associated with early cellular activity and differentiation but not bone specific
Collagen, type I, alpha 1 (COL1A1)	Associated with cell adhesion, proliferation and differentiation of the osteoblast phenotype and known as an early indicator of osteoblastic differentiation
RUNX2 (RUNX2)	Associated with osteoblastic differentiation and skeletal development
Osteocalcin (OCN)	Osteoblast specific protein, used as a marker for bone formation process (matrix synthesis and mineralization)

The 40 bone sections previously treated with ALT(14 days), GLY(14d), GLY(7d) +ALT(7d) and PBS(14d) (as described in Task 2 in **Figure 8**) were used as substrates for human stem cells (Lonza, Walkersville, MD). Prior to cell culture, the bone sections were washed with ethanol, hood dried for 30 minutes and then washed again using PBS. The sections were kept in 10% bovine fetal serum DMEM solution mix with Fungizone overnight.

The bone sections were placed in 6 well culture plates, seeded with the stem cells and cultured under conditions shown to induce osteogenic differentiation (minimal essential medium supplemented with 10% fetal calf serum, 0.1 μM dexamethasone, 10 nM β-glycerol phosphate, and 50 μg/ml ascorbic acid

phosphate). Osteogenic cell differentiation was examined by the relative expression of four osteogenic/cellular markers (i.e. alkaline phosphatase, collagen-1a1, Runx-2, osteocalcin).

Table 6. RT-PCR results (mean and standard deviation) after 7 and 14 day culture showing fold change relative to corresponding PBS control group at day 7 and 14 respectively. The results are shown in **Figure 12** and **Figure 14** as scatter and box plots. We did not measure osteocalcin at day 7 because osteocalcin does not express at early stages of differentiation.

		ALT		GLY		GLY+ALT		PBS	
		Day 7	Day 14	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
ALPH	Mean	0.863	1.484	1.784	2.501	1.592	2.358	1.038	1.032
	Std Dev	0.160	0.324	0.066	0.271	0.147	0.516	0.272	0.273
COL1A1	Mean	1.333	0.825	0.681	0.549	0.561	0.585	1.050	1.059
	Std Dev	0.370	0.155	0.101	0.226	0.127	0.086	0.370	0.353
RUNX2	Mean	1.112	0.973	1.001	1.416	0.935	1.170	1.012	1.042
	Std Dev	0.136	0.191	0.043	0.261	0.128	0.203	0.165	0.288
OCN	Mean	-	0.831	-	1.083	-	0.908	-	1.115
	Std Dev	-	0.574	-	0.206	-	0.434	-	0.653

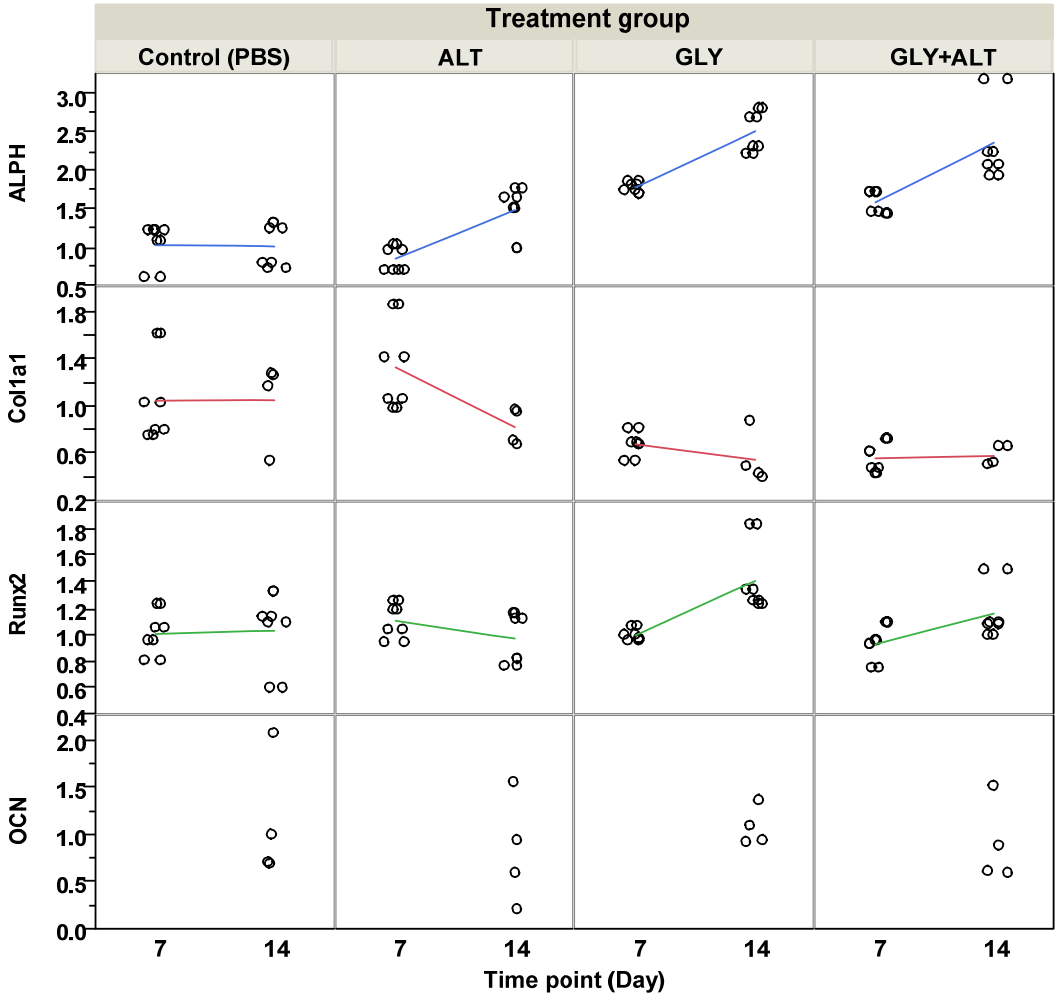

Figure 12. Temporal fold change of osteogenic markers in ALT, GLY, GLY+ALT and PBS groups at day 7 and day 14 of osteogenic culture environment. The four osteogenic markers are alkaline phosphatase (ALPH - blue), collagen 1a1 (Col1a1 - red), Runx-2 (green) and osteocalcin (OCN). The increase in ALPH and Runx2 and decrease in Col1a1 from day 7 to 14 are hallmark signs of osteoblastic differentiation. We did not measure osteocalcin at day 7 (but we did at day 14) because osteocalcin levels are undetectable in immature osteoblastic cells. Gene expression was measured, normalized to constitutive gene expression (b-actin) and expressed relative to that of PBS treated bone sections respective of the time point (day 7 or day 14 of culture respectively).

Between day 7 and day 14 of cell culture, all three treatment groups (i.e. ALT, GLY and GLY+ALT) relative to the control group (PBS) have upregulated alkaline phosphatase (ALPH) while only GLY and GLY+ALT have upregulated Runx2 (**Table 6, Figure 12**). The GLY showed highest average fold change at day 14 in both ALPH and Runx2. In col1a1 expression, ALT showed the highest rate of decrease while GLY and GLY+ALT showed significantly lower regulation of Col1a1 than the control ($p = 0.0013$ to $p = 0.0123$). We found no difference in osteocalcin expression among the groups ($p = 0.4345$ to $p = 0.9289$).

The data show that bone slices induce osteogenic differentiation of stem cells relative to that seen on plastic and that the high levels of AGE products generated by ribose treatment can further stimulate osteogenesis as measured by ALPH and Runx2 expression. The current results are largely consistent with the temporal osteogenic gene expression in osteoblasts [4, 5] where upregulation of ALPH and Runx2 and downregulations for Col1 is a hallmark of known temporal expression behavior of osteoprogenitor to pre-osteoblasts (**Figure 13**). Also the absence in detectable changes in osteocalcin expression suggests that none of the groups have yet matured beyond pre-osteoblastic stage, which is expected to occur around day 21. In conclusion, our data suggest that the current osteogenic culture was able to induce normal osteogenic differentiation in the stem cells and thus any differences in osteogenic expression between the ALT-711 treated groups and other treatment groups will likely to be due to the treatment factor.

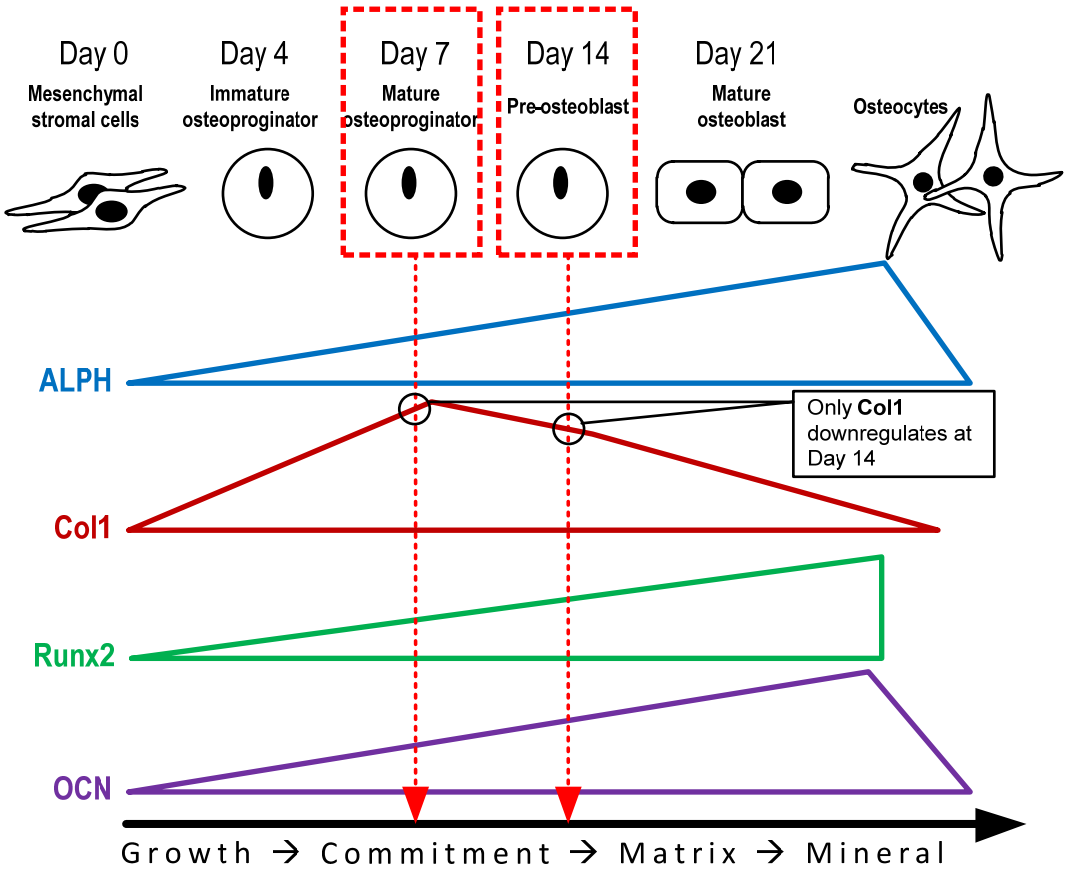

Figure 13. Temporal expression of markers during osteoblast differentiation in culture (adapted from Safadi et al 2009 [6]). While the other three markers increase gradually, Col-1 initially increases and decreases expression; we also observe this trend in **Figure 7**. However we did not observe osteocalcin as it is usually detectable only in mature osteoblast (i.e. post-day 21). **Markers**: Runx2 = RUNX2; ALPH = alkaline phosphatase, Col-1 = Collagen type I, OCN = osteocalcin.

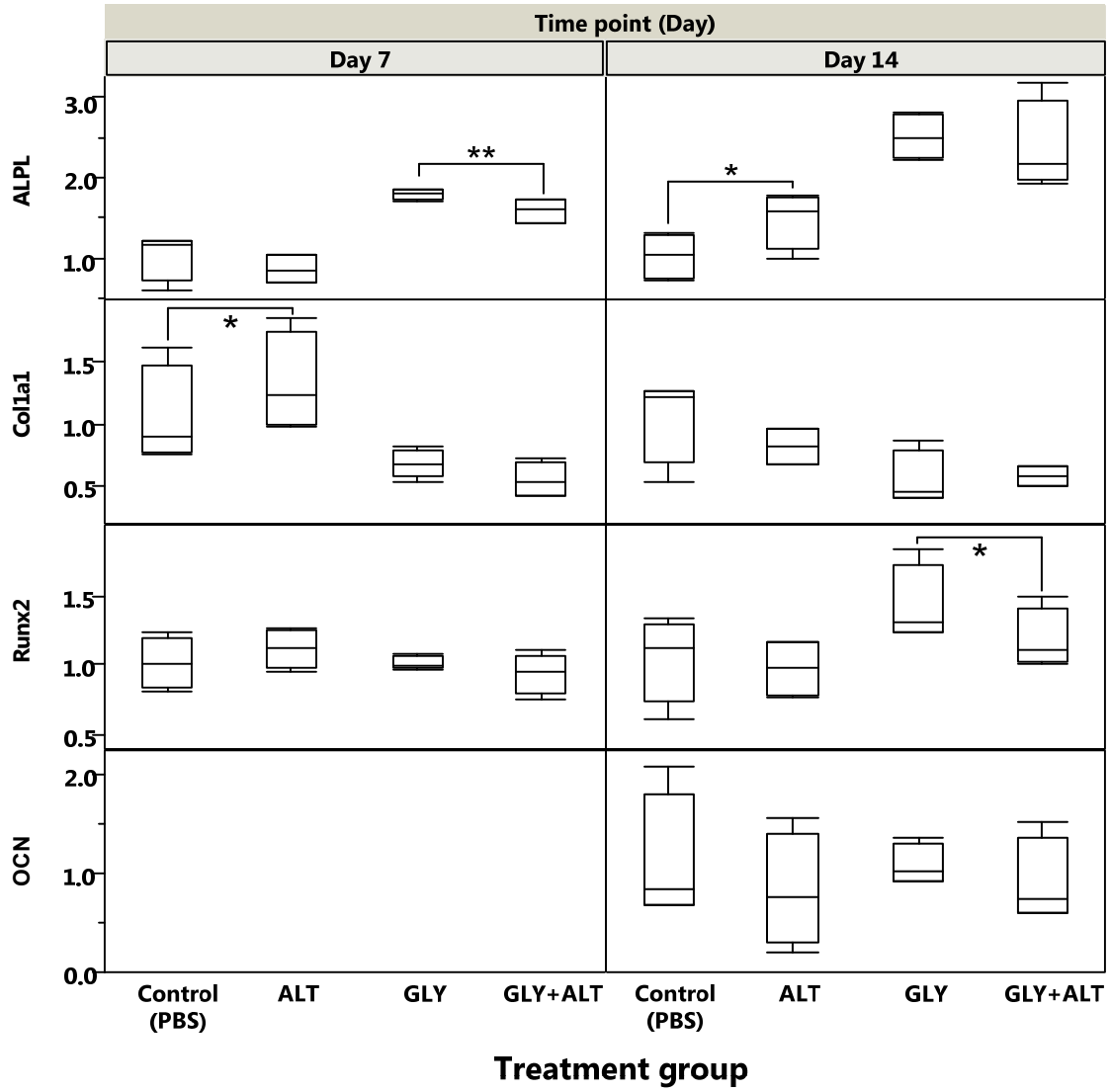

Figure 14. Boxplots of temporal osteogenic expressions grouped so that control (PBS) vs. ALT and GLY vs. GLY+ALT can be compared and potential effects of ALT on naturally (control) and artificially (GLY) glycated bone substrates can be determined. ALT was found to have significant up regulating effects on alkaline phosphatase (ALPH) at day 14 and collagen 1 (Col1a1) at day 7 in normal bone while it had down regulating effects on ALPH at day 7 and Runx2 at day 14 in artificially glycated bone. * = $p < 0.05$, ** = $p < 0.0001$

We found ALT treatment was associated with significant increases in collagen 1 (Col1a1) at day 7 and alkaline phosphatase (ALPH) at day 14 (**Figure 14**, $p = 0.0274$, $p = 0.048$ respectively), however ALT was associated with decreases in ALPH and Runx2 in the GLY+ALT group relative to GLY at day 14 ($p < 0.0001$, $p = 0.0492$ respectively). Although ALT's effect does not appear to be universal across all markers and GLY treatments, ALT at least partially promoted osteogenic expression in the bone substrates with naturally accumulated AGEs only (i.e. PBS-control) whereas it suppressed the expression markers of bone that has been artificially glycated (i.e. GLY). It is thus possible that ALT improves osteogenic activities in naturally aged bone. ALT can also suppress glycation effects on osteogeneity, consistent with the observation that it reduced AGE-fluorescence in glycated bone (**Figures 6-8**). However, it must be noted that the evidence of increased osteogenic activity when artificially glycated bone is used as substrate is in contrast to expectations from cell culture experiments that did not use bone substrates. It is possible that the type of AGE that ALT affected in naturally aged bone versus artificially aged bone could be different, however at this time we do not know why ALT have shown pro-osteogenic effects selectively. This increase of osteogenic activity with artificial glycation and its reversal with ALT requires further investigation.

In conclusion, the current stem cell culture protocol and RT-PCR method was able to replicate the temporal differentiation behavior of stem cells known from the literature. Furthermore our preliminary osteogenic data suggest that ALT can induce osteogenic effect on bone sections without any prior glycation treatment (i.e., naturally aged) and, in principal, support the basic idea of enhancing the osteogenic properties of a bone allograft using a crosslink breaker.

KEY RESEARCH ACCOMPLISHMENTS

We have identified duration and concentrations required for ALT-711 (with and without ribose) that would result detectable changes in the fluorescence levels in bone using both spectroscopy and epi-fluorescence microscopy.

We have procured 80% of tissue quota (19/24 femurs) and processed 63% of specimen requirement (15/24 femurs)

We have performed preliminary osteogenic expression tests on 60 bone sections that consistently replicated the known osteogenic behavior of bone cells on the specific substrates that are subject of this research.

We found preliminary evidences that ALT-711 can reduce levels of AGEs induced by ribose and improve osteogenic expression in untreated bone.

REPORTABLE OUTCOMES

No reportable outcome in terms of manuscripts, conference presentations or funding applied based on the work supported by this award is available at this time.

Employment or research opportunities applied for and/or received based on experience/training supported by this award

This project provided training opportunities for the following individuals whether or not they were compensated by the project funds:

1. Woong Kim, PhD: University of Auckland, New Zealand, Post-doc fellow, 2012 – present.
   RT-PCR analysis, sample procurement and preparation, fluorescence microscopy and fluorescence quantification, glycation+ALT-711 treatment

2. Richard Banglmaier, PhD: Wayne State University, Senior Research Engineer, 2012-2013.
   Fluorescence quantification using spectrophotometry

3. Daniel Oravec, MSc: Tampere University of Technology, Finland, Research Engineer, 2012 – present
   Specimen procurement, accounting, machining and mechanical test system.

CONCLUSION

Currently allograft is the only practical source of bone grafts but it carries plethora of issues such as nonunion due to poor bone quality as they are often sourced from old donors. One of main contributing factor for poor bone quality is natural accumulation of AGEs (advanced glycation endproducts) which forms non-enzymatic crosslinks that results in a brittle and biologically poor bones. It is proposed that once the crosslinks can be broken artificially by ALT-711, this will improve mechanical properties and create favorable bone matrix for the stem cells that will eventually result a better integration in the patients.

In the past period, we have laid foundational work on the project - procuring femurs, refining and making bone samples, and optimizing treatment solution concentrations and durations. Furthermore we have obtained preliminary evidence that ALT-711 can reduce level of AGEs induced by artificial glycation and induce positive osteogenic expressions in the osteoblastic stem cell culture. In the following months, we plan to complete the rest of prerequisite tasks for the main mechanical and biological tests, gather data and publish our findings.

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APPENDICES

No appendix material is included.